

TACACGTTGCGAGCGGGGACCAAGLTCGAAATAAAACGGGTGATGCTGCACCAACATGTC
TyrThrPheGlyGlyGlyThrArgLeuGluIleLeuLysArgAlaAspIleAlaProThrVal

RECOMBINANT ANTIBODY AND METHOD

The present invention relates to a humanised antibody molecule (HAM) having specificity for an antigen present on certain malignant cells and to a process for its production using recombinant DNA technology.

In the present application, the term "recombinant antibody molecule" (RAM) is used to describe an antibody produced by any process involving the use of recombinant DNA technology, including any analogues of natural immunoglobulins or their fragments. The term "humanised antibody molecule" (HAM) is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site may comprise either complete variable domains fused onto constant domains or only the complementarity determining regions grafted onto appropriate framework regions in the variable domains. The abbreviation "Mab" is used to indicate a monoclonal antibody.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')₂ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site at the end of

- 2 -

each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, have been hindered by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of monoclonal antibodies of defined specificity (1). However, most MAbS are produced by fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbS.

Since most available MAbS are of rodent origin, they are naturally antigenic in humans. Therefore, the use of rodent MAbS as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness.

There have therefore been made proposals for making non-human MAbS less antigenic in humans. Such techniques can be generically termed "humanizing" MAbS. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Some early methods for carrying out such a procedure are described in EP-A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), EP-A-0 194 276 (Celltech Limited) and WO-A-8 702 671 (Int. Gen. Eng. Inc.). The Celltech application discloses a process for preparing an

antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. It also shows the production of an antibody molecule comprising the variable domains of a mouse MAb, the CH1 and CL domains of a human immunoglobulin, and a non-immunoglobulin-derived protein in place of the Fc portion of the human immunoglobulin.

In an alternative approach, described in EP-A-87302620.7 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin using site directed mutagenesis using long oligonucleotides.

The earliest work on humanizing MABs has been carried out based on MABs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAB recognising lysozyme and a rat MAB recognising an antigen on human T cells respectively were humanized are shown by Verhoeven et al. (2) and Reichmann et al. (3).

It has been widely suggested that immunoglobulins, and in particular MABs, could potentially be very useful in the diagnosis and treatment of cancer (4,5). There has therefore been much activity in trying to produce immunoglobulins or MABs directed against tumour-specific antigens. So far, over one hundred MABs directed against a variety of human carcinomas have been used in various aspects of tumour diagnosis or treatment (6).

There have been a number of papers published concerning the production of chimeric monoclonal antibodies recognising cell surface antigens. For instance, Sahagan et al. (7) disclose a genetically engineered murine/human chimeric antibody which

retains specificity for a human tumour-associated antigen. Also Nishimura et al. (8) disclose a recombinant murine/human chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen.

According to the present invention, there is provided a humanised antibody molecule (HAM) having specificity for the TAG-72 antigen and having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domain are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin.

The variable domains of the HAM may comprise either the entire variable domains of the B72.3 MAb or may comprise the framework regions of a human variable domain having grafted thereon the CDRs of the B72.3 MAb.

The B72.3 MAb is a mouse MAb of the type IgG1-Kappa raised against a membrane-enriched extract of a human liver metastasis of a breast carcinoma (9). The B72.3 MAb has been extensively studied in a number of laboratories. It has been shown to recognise a tumour-associated glycoprotein TAG-72, a mucin-like molecule with a molecular weight of approximately 10^6 (10). Immunohistochemical studies have demonstrated that the B72.3 MAb recognises approximately 90% of colorectal carcinomas, 85% of breast carcinomas and 95% of ovarian carcinomas. However, it shows no significant cross-reactivity with a wide spectrum of normal human tissues (11 to 14).

It has surprisingly been found that humanizing the B72.3 MAb does not adversely affect its binding

activity, and this produces a HAM which is extremely useful in both therapy and diagnosis of certain carcinomas.

Preferably, the HAM of the present invention will be produced by recombinant DNA technology.

The HAM of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as the Fab or (Fab')₂ fragment; a light chain or heavy chain dimer; or any other molecule with the same specificity as the B72.3 antibody.

Alternatively, the HAM of the present invention may have attached to it an effector or reporter molecule. For instance, the HAM may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce a HAM in which the Fc fragment or CH3 domain of a complete antibody molecule has been replaced by an enzyme or toxin molecule.

The remainder of the HAM may be derived from any suitable human immunoglobulin. However, it need not comprise only protein sequences from the human immunoglobulin. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule.

According to a second aspect of the present invention, there is provided a process for producing the HAM of the first aspect of the invention, which process comprises:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy

or light chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the HAM.

The cell line may be transfected with two vectors, the first vector containing an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure as far as possible that each polypeptide chain is equally expressed.

Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially genomic DNA. Most preferably, the heavy or light chain encoding sequence comprises a fusion of cDNA and genomic DNA.

The host cell used to express the HAM of the present invention is preferably a eukaryotic cell, most preferably a mammalian cell, such as a CHO cell or a myeloid cell. It has been found, surprisingly, that the use of cDNA/genomic DNA fusions for the heavy or light chain coding sequences leads to enhanced production of the HAM of the present invention in non-myeloid mammalian cells. Thus, an important aspect of the invention is the use of such fusions in non-myeloid mammalian cells in order to express the HAM.

The present invention also includes cloning and expression vectors and transfected cell lines used in the process of the invention, therapeutic and diagnostic compositions containing the HAM of the invention and uses of such compositions in therapy and diagnosis.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 15 and 16.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 shows the DNA sequences encoding the unprocessed variable regions of the B72.3 MAb obtained by sequencing the cDNA clones pBH41 and pBL52. Panel A shows the sequence coding for the VH region and the predicted amino acid sequence. Panel B shows the sequence coding for the VL region and the first 21 residues of the CL region, together with the predicted amino acid sequence. The points of fusion with the human C regions are indicated with arrows.

The putative sites of cleavage of the signal peptide are indicated by open triangles. The numbers refer to the nucleotides in the original cDNA clones;

Figure 2 is a schematic diagram of the construction by site directed mutagenesis, restriction and ligation of the chimaeric heavy chain gene;

Figure 3 is a schematic diagram of the construction by partial restriction and ligation of the chimaeric light chain gene;

(In Figures 2 and 3, coding sequences are shown as boxes, dark for the variable regions and light for the constant regions. Restriction enzymes are abbreviated as follows: EcoRI=E; BglII=B; HindIII=H; MboII=M; HpaI=Hp; and ScaI=Sc. Dotted lines indicate the continuation of the sequence into vector or constant region DNA.)

Figure 4 is a schematic diagram of the hCMV expression vector and the four alternative cDNA or gene constructs inserted into the EcoRI site. The chimaeric heavy chain gene was inserted using a BamHI-EcoRI oligonucleotide adapter. Coding sequences are represented by boxes, dark for the variable regions and light for the constant regions. The direction of transcription is indicated with an arrow;

Figure 5 shows an ELISA analysis of COS-cell transfectant supernatants. The level of antigen-binding capacity in the supernatant of COS-cell transfectants was analysed as described later. Dilution curves were plotted out against the optical density of the colour change. Different antibodies were used to recognise mouse or human epitopes, and consequently the antigen-binding levels for each curve are not strictly comparable. Each

curve represents a co-transfection, as follows:
△ mouse heavy chain, mouse light chain; ▲ mouse heavy chain, chimaeric light chain; □ chimaeric heavy chain, mouse light chain; ■ chimaeric heavy chain, chimaeric light chain;

Figure 6 shows SDS-PAGE analysis in a reducing gel of immunoprecipitations from the supernatants of transfected COS-cells. The DNA used for the transfection was as follows: Lane 1, mouse light chain alone; Lane 2, mouse light chain, mouse heavy chain; Lane 3, mouse light chain, chimaeric heavy chain; Lane 4, chimaeric light chain alone; Lane 5, chimaeric light chain, mouse heavy chain; and Lane 6, chimaeric light chain, chimaeric heavy chain. The antibodies used for the immunoprecipitations were: Lanes 1-3, rabbit anti-mouse F(ab')₂; Lanes 4-6, rabbit anti-human F(ab')₂;

Figure 7 shows SDS-PAGE analysis of immunoprecipitations from the supernatants of transfected COS-cells, under non-reducing (lanes 1-3), and reducing (lanes 4-6) conditions. The DNA used for transfection was as follows: lanes 1 and 4, chimaeric light chain clone; lanes 2 and 5, chimaeric light chain, mouse heavy chain; lanes 3 and 6, chimaeric light chain, chimaeric heavy chain. The antibody used for the immunoprecipitation in each case was rabbit anti-human F(ab')₂;

Figure 8 shows SDS-PAGE analysis on a reducing gel of immunoprecipitations from the supernatants of transfected COS-cells, grown and labelled in the absence (lanes 1 and 3), and the presence (lanes 2 and 4) of tunicamycin. The DNA used for the transfections was as follows: lanes 1 and 2, chimaeric light chain clone; and lanes 3 and 4, chimaeric light chain and chimaeric heavy chain. The

antibody used for immunoprecipitation in each case was rabbit anti-human F(ab')₂ ;

Figure 9 shows reducing and non-reducing SDS-PAGE gels of chimeric B72.3 produced by CHO cells;

Figure 10 shows a two dimensional SDS-PAGE gel of chimeric B72.3 produced by CHO cells;

Figure 11 shows a time course study of tumour labelling using B72.3 antibodies;

Figure 12 shows the tissue/tumour ratio of the B72.3 antibodies; and

Figure 13 shows the construction of plasmid TR002

EXAMPLE 1

Molecular cloning and sequencing of the B72.3 heavy and light chain cDNAs.

Polyadenylated RNA was isolated from the B72.3 hybridoma cell line using the guanidinium isothiocyanate/caesium chloride method (15). Double stranded cDNA was synthesised (17) and a cDNA library was constructed in bacteriophage λ gt 10 vector using EcoRI linkers (18). Two screening probes were synthesised, complementary to mouse immunoglobulin heavy and light chain constant regions. The heavy chain probe was a 19 mer complementary to residues 115-133 in the CH1 domain of the mouse γ 1 sequence (19). The light chain probe was a 20 mer complementary to residues 4658-4677 of the genomic mouse CK sequence (20). The probes were radio-labelled at the 5' terminus with [γ -³²P] ATP using T4 polynucleotide kinase (Amersham International) and used to screen the cDNA library.

Clones which contained the complete leader, variable and constant regions of both the heavy and

light chains were isolated. The EcoRI cDNA inserts were subcloned into M13mp8 vectors for sequencing (21), generating a heavy chain clone, designated pBH41, and a light chain clone, designated pBL52. Nucleotide sequence analysis was carried out according to the chain termination procedure (22).

The 980 base pair EcoRI insert in pBL52 was fully sequenced (22). The EcoRI insert in pBH41 was shown to comprise approximately 1700 base pairs by agarose gel electrophoresis. The variable domain and the 5' region of the CH1 domain were sequenced, as was the 3' end of the clone to confirm the presence of the correct mouse χ 1 termination sequences. The DNA and predicted amino acid sequences for the unprocessed variable regions of pBH41 and pBL52 are shown in Figure 1. Examination of the derived amino acid sequence revealed considerable homology with other characterised immunoglobulin genes, and enabled the extent of the leader, variable and constant domains to be accurately determined. In addition, MAb B72.3 was confirmed to be an IgG1 K antibody, as previously reported (9).

Construction of the Chimaeric Mouse-Human Heavy Chain Clone

A genomic clone containing sequences coding for the human C χ 4 region was isolated as a HindIII fragment from the cosmid COS Ig8 (23) and then cloned via pAT153 into M13tg130 as an EcoRI-BamHI fragment to form pJA78. Following DNA sequence analysis, an 18 mer oligonucleotide was synthesised and site specific mutagenesis was performed to convert a C residue to an A residue, thereby generating a novel HindIII site at the start of the CH1 exon, to yield pJA91.

- 12 -

Site directed mutagenesis was performed (24) using EcoRI- and BglI-cut M13mp18 to generate a gapped duplex with the relevant phage template. DNA was transformed into E. coli HB2154 and resultant transformants were propagated on E. coli HB2151 (Anglian Biotechnology Ltd) as described in the protocols provided. All mutations were sequenced using the chain termination procedure (22). All sequenced fragments were subsequently recloned into other vectors in order to exclude the possibility of secondary mutations which may have occurred during the mutagenesis procedure.

The VH domain from the B72.3 heavy chain cDNA, cloned in M13mp9 as pBH41, was isolated as an EcoRI-BglI fragment and introduced into the EcoRI-HindIII sites of pJA91 in conjunction with a 32 base pair BglI-HindIII adaptor to yield pJA93. The product was therefore a chimaeric immunoglobulin heavy chain gene containing a variable region derived from a mouse cDNA clone fused to a sequence, comprising the CH1, H, CH2 and CH3 domains separated by introns, derived from a human genomic clone. The accuracy of the variable/constant region junction was confirmed by nucleotide sequence analysis. A schematic diagram of details of the construction is given in Figure 2. The γ_4 constant region was selected as it possesses a limited repertoire of effector functions, but does bind to staphylococcal protein A, a potentially useful reagent for purification.

Construction of the Chimaeric Mouse-Human Light Chain Gene

The mouse light chain cDNA clone, pBL52, contains a cutting site for MboII 18 base pairs

- 13 -

downstream from the junction of the variable and constant domains. Due to sequence homology between the mouse and human CK genes, an identical cutting site exists in the latter gene (25) and use of this site provides a method of fusing the mouse variable and human constant domains. Partial digestion of the EcoRI fragment containing the mouse cDNA clone with MboII generated a 416 base pair EcoRI-MboII fragment with a single residue overhang. A genomic clone, comprising an M13-derived vector containing the human C-kappa gene on a PstI-HindIII fragment was digested with FokI. A 395 base pair fragment containing the majority of C-kappa was cloned into pAT153 using EcoRI linkers to form pNW200. Digestion of a 945 base pair ScaI-HindIII fragment from pNW200 with MboII generated a 374 base pair MboII-HindIII fragment, which could anneal with and be ligated to the 416 base pair EcoRI-MboII fragment described above. The two fragments were ligated into a pSP64 vector linearised with EcoRI and HindIII, and used to transform competent E. coli HB101. The variable/constant region junction was sequenced in order to confirm the correct fusion. The construction is outlined schematically in Figure 3.

Construction of Expression Vectors for Transient Expression in COS Cells

The heavy and light chain chimaeric genes, as well as the mouse heavy and light chain cDNA clones, were inserted separately into the unique EcoRI site of plasmid pEE6 (27). The light chain encoding plasmid was designated EE6.cL.neo. For the chimaeric heavy chain, this was accomplished by using an oligonucleotide adapter to change the 3' BamHI site

to an EcoRI site to give an EcoRI fragment for cloning. The heavy chain encoding plasmid was designated EE6.CH.gpt. This plasmid contains the strong promoter/enhancer and transcriptional control element from the human cytomegalovirus (hCMV) inserted into a unique HindIII site upstream of the EcoRI site. In addition, an SV40 origin of replication site is provided by the SV40 early promoter which drives a selectable marker gene, either a neomycin-resistance gene (neo) for light chain genes or a guanine phosphoribosyl transferase gene (gpt) for heavy chain genes, inserted into a unique BamHI site. The plasmid also contains an ampicillin-resistance gene allowing selection and propagation in bacterial hosts. The structures of the expression vector and immunoglobulin gene inserts are shown schematically in Figure 4.

Transfections and ELISA Analysis of Antibody Production

The four expression constructs described above were used singly or in heavy/light chain gene pairs to transfect COS-1 cells (26). The cells were left to incubate in DNA-DEAE dextran solution for six hours, then shocked for two minutes with 10% DMSO in HEPES-buffered saline. The cells were washed and incubated in medium containing 10% foetal calf serum for 72 hours.

Following incubation at 37°C for 72 hours the cell supernatants and lysates were analysed by ELISA for heavy and light chain production and binding of antigen.

The medium (500 μ l per 10^5 cells) was removed for ELISA analysis. Cell lysates were prepared by

- 15 -

lysis of 10^5 cells in 500 μ l 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01M sodium phosphate pH 7.5, 0.1M sodium chloride and 0.001M EDTA. Lysates and conditioned medium were centrifuged for 5 minutes in an Eppendorf centrifuge to remove nuclei and cell debris, and stored at 4°C before analysis.

Microtitre plates were coated with 0.25 μ g per well of sheep or goat antibody reactive against either human or mouse specific epitopes on the heavy or light chains. Supernatants or lysates from transfected COS cells were diluted 1:2 or 1:4 respectively in sample conjugate buffer containing 0.1M Tris-HCl pH 7.0, 0.1M sodium chloride, 0.02% Tween 20 and 0.2% casein. 100 μ l of each diluted sample were added to each well and incubated for 1 hour at room temperature with gentle agitation. Following washing six times with wash buffer (phosphate buffered saline containing 0.2% Tween 20, pH 7.2), 100 μ l of 1:5000 dilution of standard horseradish peroxidase - conjugated antibody reactive against either human or mouse specific epitopes were added per well. The plates were incubated for 1 hour at room temperature, and then washed six times with wash buffer. 100 μ l of substrate buffer containing 0.1 mg/ml tetramethylbenzidine (TMB), 0.1M sodium citrate, pH 6.0 and 0.005% H_2O_2 were added to each well to generate a colour change. The reaction was terminated after 2-3 minutes by adjusting the solution to pH 1.0 with 1.5M sulphuric acid. The optical density was determined at 450nm for each well by measurement in a Dynatech laboratories MR600 microplate reader. Standard curves were generated using known concentrations of the appropriate human or mouse immunoglobulins.

- 16 -

Antigen binding assays were performed in an analogous manner. Microtitre plates were coated with 0.25 µg per well of purified TAG-72 antigen (6) obtained from human patients or from tumour xenografts implanted in nude mice (both gifts of J. Schlom, MCI). Following washing six times in wash buffer, samples from COS-cell transfections were added as previously, and the same subsequent procedures carried out, using goat anti-mouse or human F(ab')₂ linked to HRP as the second antibody.

A number of assay systems using different capture antibodies were developed and cross-correlated to investigate the potential products of each transfection. In all cases, mouse light chain and chimaeric light chain were detected in the supernatants and lysates of appropriately transfected cells. However heavy chains were only detected in the supernatants when co-transfected with light chain. A low level of heavy chain was detected in the cell lysate in each case, supporting a suggestion of inhibition of heavy chain secretion in the absence of light chain.

Assembly assays, which detect the presence of associated polypeptide chains, demonstrated the formation of multimers containing at least one heavy and one light chain when both genes were co-transfected. Mouse genes and chimaeric genes appeared equally capable of assembly and formation of hybrid molecules.

Antigen binding analysis (see above) demonstrated that the mouse heavy and light chain co-transfections generated an antibody molecule capable of recognising antigen. Replacement of the mouse gene for either chain with the appropriate chimaeric gene led to the production of a hybrid

- 17 -

molecule with antigen binding specificity in the ELISA assays. Finally, transfection of the COS cells with both the chimaeric heavy and light chain genes generated a complete chimaeric antibody molecule with antigen binding specificity. The ELISA data from one experiment are presented in Figure 5. These experiments demonstrate that "humanisation" of the antibody molecule does not have a significant effect on its antigen recognition capability.

Immunoprecipitation of Antibody Molecules from
Biosynthetically Labelled COS-Cell Transfectants

Preliminary experiments suggested that there was little expression from the transfected DNA in the initial 24 hours. Accordingly following transfection, COS cells were allowed to recover for 24 hours in DMEM containing 10% foetal calf serum. The medium was then replaced with methionine-free DMEM, to which [35 S] methionine (NEN) had been added at 200 μ Ci/ml. The cells were metabolically labelled for 48 hours, and conditioned media and cell lysates prepared as previously.

Analysis by reducing SDS-PAGE of aliquots of COS cell supernatant demonstrated the appearance of labelled immunoglobulin protein without further purification, while use of Protein A-Sepharose was shown to selectively precipitate whole antibody, but not light chain alone, from the COS-cell supernatant.

Further analysis of the assembly and secretion of antibody molecules was performed by immunoprecipitation using anti-human F(ab')₂ and anti-human C-kappa anti-sera bound to Protein A-Sepharose. Affinity-purified rabbit antibodies against human IgG F(ab')₂ and human K chain were

- 18 -

used for immunoprecipitations, following coupling to Protein A - Sepharose. Both cytoplasmic and secreted antibodies were analysed on an SDS-10% PAGE system under reducing and non-reducing conditions. The gel was treated with an autoradiography enhancer, dried and exposed to Fuji RX film. The results are shown in Figure 6.

Both antisera immunoprecipitated proteins with an apparent molecular weight of 55K and 28K, which coincided with the positions of the Coomassie-stained immunoprecipitating heavy and light immunoglobulin chains respectively. The use of the latter antisera demonstrated that light chain is found associated with heavy chain in the COS-cell supernatants. A comparison of immunoprecipitations analysed by reducing and non-reducing SDS-PAGE (See Figure 7) suggests that the heavy and light chains are assembled as the correct tetrameric molecule. In addition there is evidence for the secretion of free light chain dimers and partially assembled heavy and light chain multimers.

Due to the presence of secondary structure caused by disulphide bonds, the mobility of the immunoglobulin chains is different in the two systems. In order to analyse the presence and potential role of glycosylation, COS cells were treated with tunicamycin, at the same time as the radioactive label was added. To inhibit N-linked glycosylation, COS cells were grown in medium containing 10 µg/ml tunicamycin diluted from a stock solution. To ensure that the pool of lipid-linked oligosaccharide was depleted, the cells were maintained in the tunicamycin-containing medium for 2 hours prior to addition of radioactive label.

Following immunoprecipitation the protein products were analysed by SDS-PAGE, as shown in Figure 8. It is clear from the decrease in apparent molecular weight, from 55K to 52K, that the chimaeric heavy chain, but not the light chain, undergoes N-linked glycosylation. When the glycosylation is inhibited, the protein is still secreted, although the level of expression appears to be decreased.

These results demonstrate that each of the immunoglobulin genes is correctly transcribed and translated. The two mouse genes and the chimaeric light chain are cDNA-like, while the chimaeric heavy chain gene possesses characteristics of both cDNA and genomic DNA. Both types of construct appear to be expressed at a similar level and with similar fidelity. It is clear therefore that transcript splicing occurs where necessary, but it is not an obligatory requirement for correct expression of immunoglobulin genes in COS-cells.

The expressed heavy and light chains associate in the correct manner, presumably limited by the availability of free polypeptide chain. Mouse and human polypeptide sequences appear interchangeable in the association of heavy and light chains. The product is an assembled tetrameric antibody molecule, which is expressed at a high level, glycosylated and secreted into the culture medium.

Development of Stable Cell Lines in CHO Cells

Stable Light Chain Producing Cell Line

Chinese hamster ovary (CHO-K1) cells were grown in attached culture in Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal calf serum (FCS),

non-essential amino acids (NEAA) and glutamine (2mM). A confluent culture was trypsinised, the cells washed once in phosphate buffered saline (PBS) and resuspended at 10^7 cells/ml.

DNA from plasmid EE6.cL.neo, comprising the chimaeric light chain gene expressed from the HCMV promoter, was digested with SalI to generate linear molecules and then ethanol precipitated. The precipitate was resuspended in PBS and 40 μ g of DNA was added to 10^7 CHO-K1 cells in buffer at 4°C. The DNA was introduced into the cells by electroporation, in which the cell suspension and DNA were treated with two pulses of 2000 volts. After electroporation the cells were returned to 4°C for 10 min prior to plating out at a density of 5×10^5 cells per 90mm Petri dish in DMEM growth medium containing supplements.

Following incubation at 37°C overnight, selection for introduced DNA was applied by adding G418 to a final concentration of 1mg/ml. Resistant colonies were observed after 10-14 days incubation in selective medium.

Resistant colonies were picked from the transformation plates using filter paper squares soaked in trypsin solution and transferred into individual wells of 24 well tissue culture plates. The culture medium from the wells was assayed for the presence of chimaeric light chain using an ELISA assay and cell lines secreting light chain were identified. Lines producing light chain at levels between 100ng/ml and 16 μ g/ml were identified. Those producing the highest amounts were cloned out by the limiting dilution method. One such clone, cL18, was used for subsequent studies.

Stable Chimaeric Antibody Producing Cell Line

DNA from plasmid EE6.ch.gpt (also designated as JA96), comprising the chimaeric heavy chain gene expressed from the HCMV promoter, was digested with Sall to generate linear molecules and then ethanol precipitated. CHO-cl18 cells producing chimaeric light chain were prepared for electroporation as described above, and the JA96 DNA (40 µg per 10⁷ cells) was introduced by electroporation. Following overnight incubation in non-selective medium, selection for resistance to mycophenolic acid was applied. Selective medium comprised DMEM, 10% FCS, NEAA, glutamine, xanthine, hypoxanthine, thymine and mycophenolic acid (10 µg/ml). Resistant colonies were detected after 10-14 days and these were picked into 24 well plates as described above. Antibody producing cell lines were identified using an antigen binding assay based on the antigen TAG-72 recognised by the antibody B72.3. Cell lines producing antibody at levels ranging from 0.1-40 µg/ml were isolated. Two of these cell lines, F6 and F11, were used in further studies.

Purification of Chimaeric Antibody

Chimaeric B72.3 was purified from CHO cell culture supernatant by affinity chromatography using Protein-A Sepharose and ion-exchange chromatography. Cell culture supernatant was adjusted to pH 8.8 with sodium glycinate (0.2M) and applied to a column of Protein-A Sepharose pre-equilibrated with glycine/glycinate buffer at pH 8.8 (50mM). After sample loading, the column was washed with equilibration buffer and the antibody eluted with a

gradient of decreasing pH made up of disodium hydrogen phosphate (0.2M) and citric acid (0.1M). Fractions containing chimaeric antibody were pooled, dialyzed into 50 mM phosphate buffer pH 8.0 and then applied to a column of DEAE-Sepharose pre-equilibrated with 50mM phosphate buffer pH 8.0. The column was washed with equilibration buffer and elution of antibody achieved with a linear gradient of sodium chloride from 0.0 to 0.2M. Purified antibody was then dialyzed into an appropriate buffer for the intended use, e.g. PBS for animal studies, and concentrated by ultrafiltration. Typical yields of chimaeric antibody were 20 mg per litre of starting supernatant.

Purity and assembly of the chimaeric antibody was tested by SDS-polyacrylamide gel electrophoresis (PAGE), both reducing and non-reducing (Fig 9), and by HPLC gel filtration.

N-terminal amino acid sequencing of the antibody revealed a single sequence encoding light chain, which corresponded exactly to the expected amino acid sequence deduced from the DNA sequence. The heavy chain was N-terminally blocked and not amenable to amino acid sequencing. Antigen binding activity was demonstrated in an ELISA format assay.

Chimaeric B72.3 made in COS or CHO cells contains a proportion of material (10-20%) which fails to form an inter-heavy chain disulphide bridge but otherwise assembles correctly into 150kD molecules containing two heavy and two light chains. This material is present in antibody when secreted from the cell and co-purifies with antibody in which the inter-heavy chain disulphide bridges have formed. This appears to be a common property of human IgG4 molecules and occurs with all molecules of

this type analysed to date, including a mouse-human IgG4 chimaeric anti-NP antibody and two different IgG4 myeloma proteins.

On non-reducing SDS-polyacrylamide gel electrophoresis of chimaeric B72.3, two bands are seen, one at the expected size of 150 kD and one of about 80 kD (Fig. 9). Both of these bands contain intact heavy and light chains as shown by non-reducing/reducing two dimensional SDS-PAGE (Fig. 10). Reducing SDS-PAGE shows only intact heavy and light chain (Fig. 9). Native (non SDS) electrophoresis and HPLC gel filtration show only one species corresponding to 150 kD material. Thus the about 80 kD band seen on non-reducing SDS-PAGE represents materials with a molecular weight of 150 kD in solution. The two halves of the molecule are only separated when other inter-heavy chain interactions are disrupted, e.g. when run on non-reducing SDS-PAGE

Efficacy Studies

The chimaeric B72.3 antibody is capable of being used to advantage in a number of circumstances. For example, after suitable labelling by radioisotopes or other detection procedure, the antibody can be demonstrated to locate and bind in vivo to solid tumours where some or all of the tumour cells express the specific antigen TAG-72. The experiment described below is one example of the ability of the chimaeric antibody to locate human tumour cells bearing the specific antigen, in this case in a nude mouse model system.

Chimaeric B72.3 and mouse B72.3 antibodies were radioactively labelled with ^{125}I by the Chloramine

T method to an approximate specific activity of 5 $\mu\text{Ci}/\mu\text{g}$. Groups of 4 female nude mice bearing subcutaneous LS174T xenografts on the flank were injected intravenously with 100 μCi of either Chimaeric B72.3 or mouse B72.3 in 0.1ml PBS. Groups of animals were sacrificed at intervals for the collection of tissue samples, which were weighed, dissolved in 7M potassium hydroxide and counted in an LKB model 1270 "Rackbeta" counter. Tissue uptake was calculated as the mean percentage of injected dose per gram of tissue from a group of four animals.

Fig. 11 shows a time course study of the mouse and chimaeric antibodies and demonstrates clearance of the antibodies from the blood pool and uptake at the tumour site. The chimaeric antibody appears to clear somewhat faster from the blood pool but locates to the tumour adequately with approximately the same profile as the mouse antibody. This sample data suggests that the novel engineered antibody is functional *in vivo*.

Fig. 12 shows the tumour to tissue ratio at 24, 48 and 168 h. It can be seen that tumour/tissue ratios increase with time and that in this model system the chimaeric antibody has a superior tumour/tissue ratio compared to the mouse antibody.

EXAMPLE 2

Chimaeric B72.3 - Other IgG Isotypes

Construction of Chimaeric Antibody Genes

Assembly of Chimaeric Antibody Genes

Genomic DNA sequences containing the human IgG1, 2 and 3 genes were isolated from larger DNA

- 25 -

inserts in phage λ and were introduced into phage M13 via pJA103 which contains the human IgG4 gene with a HindIII site at the 5' end of the CH1 exon and a BamHI 3' to the CH3 exon. The M13 vector is M13tg130 which has two amber mutations in essential genes and is therefore suitable for high efficiency site-directed mutagenesis experiments using the procedures described earlier. A HindIII site was introduced at the 5' end of the CH1 exon in each isotype gene to give pRB11 (IgG1), pRB14 (IgG2) and pRB16 (IgG3). SalI and BglII sites were also introduced into pRB11 towards the 3' end of the CH1 exon and towards the 3' end of the intron following the CH1 exon respectively. The isotypes were then reisolated as HindIII-BamHI fragments and sub-cloned into pAT153 to give RB18 (IgG1), RB26 (IgG2), and RB20 (IgG3). The B72.3 VH DNA sequence was isolated and was ligated to the linking oligonucleotide which was used earlier to make the IgG4 chimaeric heavy chain gene so as to give an EcoRI-HindIII VH fragment. This fragment was ligated to the human IgG1 HindIII-BamHI containing fragment of RB18 and cloned in pAT153 to give pRB22. To construct the chimaeric B72.3 VH, the VH fragment described above was ligated to the HindIII-BamHI fragment of pRB26 and recloned in pAT153 to give pRB27.

To construct the chimaeric B72.3 VH/IgG3 gene, the VH fragment described above was ligated to the HindIII-BamHI fragment of pRB20 and recloned in pAT153 to give pRB23.

Assembly of Genes in Expression Vectors

The chimaeric genes were isolated as EcoRI-BamHI fragments from pRB22, 27 and 23 described

- 26 -

above and cloned between the EcoRI and BclI sites of JA96, the B72.3 IgG4 chimaeric heavy chain expression vector, thus replacing the IgG4 chimaeric gene. The resultant chimaeric expression plasmids were named RB24 (IgG1 chimaera), RB28 (IgG2 chimaera) and RB25 (IgG3 chimaera).

Demonstration of production, assembly and activity were performed as in Example 1.

EXAMPLE 3

Chimaeric B72.3 IgG4 F(ab')₂

Construction of F(ab') Heavy Chain Gene

Assembly of F(ab') Gene

pJA79 is an M12tg130 vector which contains the human IgG4 heavy chain gene modified so that the sequence from the first nucleotide after the last codon of the hinge exon to the last nucleotide of the CH3 domain inclusive has been removed by oligonucleotide directed site specific deletion. The hinge and 3' untranslated region and part of the M13 sequence can be isolated as a 1.1 kbp BglII fragment. This fragment can be used to replace the analogous fragment in the full length B72.3/IgG4 chimaeric heavy chain gene clone pJA93 to give plasmid JA94 which therefore contains a chimaeric gene potentially capable of being expressed to produce a B72.3 IgG4 chimaeric F(ab') heavy chain protein.

Assembly of Gene in Expression Vector

Plasmid pJA94 described above was used to recover the F(ab') gene as an EcoRI-BamHI 1475 bp fragment. This fragment was cloned into the unique EcoRI site of the pEE6 expression vector using a BamHI to EcoRI oligonucleotide adapter to give pJA97.

Test of Genes in Cos Cells

The chimaeric F(ab') gene in pJA97 was expressed in COS cells in conjunction with a suitable construct capable of expressing of chimaeric light chain polypeptide as described above. PAGE analysis of the expression products and subsequent inspection of the DNA sequence of the CH1-hinge intron suggested that splicing out of the intron was not occurring correctly leading to the production of an aberrant heavy chain polypeptide.

Reconstruction of IgG4 F(ab') Heavy Chain Gene

Assembly of F(ab') Gene

pJA94 described above was derived from pJA93 which in turn was derived from pJA91. This clone was initially an M13tg130 based vector, i.e. an amber phage capable of being used in the efficient gap-heteroduplex mutagenesis procedure described earlier. In order to repeat the mutagenesis procedure at high yield, the chimaeric F(ab') heavy chain gene was isolated as an EcoRI fragment and recloned into M13tg130 to give pJA100. By oligonucleotide directed site specific mutagenesis, a SalI site was introduced towards the 3' end of the

- 28 -

CH1 exon to give pJA108. The introduced SalI site in the CH1 domain codes for the fifth and fourth from last amino acids of the CH1 domain. To reconstruct the hinge into the end of the CH1 domain, four oligonucleotides were made which together are able to code for the last five amino acids of the CH1 domain, the hinge sequence, two in-frame stop codons and an EcoRI site.

The oligonucleotides were assembled and cloned into M13 and mp11 between the SalI and EcoRI sites in the polylinker, sequenced, reisolated and ligated to the gene containing the EcoRI-SalI 700bp fragment from pJA108 to reconstruct the chimaeric B72.3 F(ab') heavy chain gene.

The reconstructed CH1/hinge sequence should be:

CH1 hinge

Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro
Ser Cys Pro Stop

The oligonucleotide used to form this CH1/hinge were

1. 5TCGACAAGAGAGTTGAGTCCAAATATGGG
2. 3'GTTCTCTCAACTCAGGTTTATACCCGGGGG
3. 5'CCCCCGTGCCCATCATGCCCATGATG
4. 3'CACGGGTAGTACGGGTACTACTTAA

In the vector, oligonucleotides 1 and 3 produced the sense strand and oligonucleotides 2 and 4 produced the anti-sense strand.

- 29 -

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI vector fragment of pJA96 to give pJA114.

Test of Genes in COS Cells

The genes were tested in COS cells as described above. On non-reducing SDS-PAGE the material appeared to be produced as F(ab') material only. Reducing SDS-PAGE showed the presence of light chain and truncated heavy chain equivalent to that expected from the F(ab') gene.

Development of Stable Cell Lines in CHO Cells

The expression plasmid pJA114, comprising the B72.3 chimaeric F(ab') heavy chain gene fragment expressed from the HCMV promoter, was introduced by electroporation into the CHO cell line CL18 described above. The procedure was similar to that described for introduction of the full length chimaeric heavy chain except that the SalI digestion was omitted and the DNA was introduced as closed circular DNA. Cell lines resistant to mycophenolic acid and expressing function F(ab') antibody were identified by screening culture supernatants in an antigen binding ELISA format assay as described earlier. Cell lines expressing between 0.1-6 µg/ml F(ab') were isolated. One cell line, FB9 was used for further studies.

- 30 -

Purification of Chimaeric F(ab')₂ Antibody

Chimaeric F(ab') was purified from CHO cell supernatant using immunopurification. An immunopurification reagent was prepared by linking NH3/41, an antibody with specificity for human Kappa chain sequence, to cyanogen bromide activated Sepharose by standard methodology. This material was packed into a column and equilibrated with PBS. CHO cell culture supernatant containing chimaeric F(ab') was applied to the column and the column was washed with PBS. Elution of chimaeric F(ab') was then achieved using 4.5M guanidine hydrochloride. Fractions containing chimaeric F(ab') were then dialyzed extensively into PBS and concentrated by ultrafiltration.

Purity and assembly of F(ab') was tested by SDS-PAGE (both reducing and nonreducing and by HPLC gel filtration. Antigen binding activity was demonstrated using an ELISA format assay. Approximately 10% of the material can be found as F(ab')₂ which forms without further treatment.

EXAMPLE 4Chimaeric B72.3 IgG1 F(ab')₂Construction of F(ab') Heavy Chain Gene

Example 2 discloses the vector RB22 which contains the B72.3/human IgG1 chimaeric cloned gene in pAT153. Vectors JA96 and JA108 are mentioned above. The plasmid TR002 containing hinge modified gene was constructed as shown in Figure 13. The

chimaeric F(ab') region containing the B72.3 VH/IgG1 was isolated as a 0.7 kbp fragment from RB22 by treating the DNA with Sall, removing the 5' phosphate from the Sall site with calf intestinal phosphatase (CIP) and recutting the DNA with EcoRI.

The IgG1 hinge was assembled by kinase labelling 500pm of top and bottom strand oligonucleotide and annealing the oligonucleotides by heating to 70°C and cooling to room temperature in the kinase buffer. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above, and the CIP'ed 5' ends were kinased.

Assembly of Gene in Expression Vector

The chimaeric B72.3 IgG1 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector fragment of JA96 to give TR002. Expression of TR002 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3 will produce material which should assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')₂.

Thus, it has been demonstrated that it is possible to produce a HAM having specificity derived from a mouse MAb but having human constant regions, and which may have an important note to play in cancer diagnosis and therapy.

It will be appreciated that the present invention has been described above by way of illustration only, and that variations or modifications of detail can be made without departing from the scope of the invention.

References

1. Kohler & Milstein, Nature, 265, 495-497, 1975.
2. Verhoeyen et al., Science, 239, 1534-1536, 1988.
3. Reichmann et al., Nature, 332, 323-324, 1988.
4. Ehrlich, P., Collected Studies on Immunity, 2, John Wiley & Sons, New York, 1906.
5. Levy & Miller, Ann.Rev.Med., 34, 107-116, 1983.
6. Schlom & Weeks, Important Advances in Oncology, 170-192, Wippincott, Philadelphia, 1985.
7. Sahagan et al., J. Immunol., 137, 3 1066-1074,
8. Nishimura et al., Cancer Res., 47 999-1005, 1987.
9. Colcher et al., PNAS, 78, 3199-3203, 1981.
10. Johnson et al., Cancer Res., 46, 850-897, 1986.
11. Stramignoni et al., Int.J.Cancer, 31, 543,552, 1983.
12. Nuti et al., Int.J.Cancer, 29, 539-545, 1982.
13. Thor et al., J.Nat.Cancer Inst., 76, 995-1006, 1986.
14. Thor et al., Cancer Res., 46, 3118-3124, 1986.
15. Maniatis et al., Molecular Cloning, Cold Spring Harbor, New York, 1982.

16. Primrose and Old, Principles of Gene Manipulation, Blackwell, Oxford, 1980.
17. Gubler and Hoffman, Gene, 25, 263-269, 1983.
18. Huynh et al. Practical Approaches in Biochemistry IRL, Oxford (Ed. Glover, M.M.), 1984.
19. Honjo et al., Cell, 18, 559-568, 1979.
20. Max et al., J. Biol. Chem., 256, 5116-5120, 1981.
21. Messing & Vieira, Gene, 19, 269-276, 1982.
22. Sanger et al., PNAS, 74, 5463-5467, 1977.
23. Krawinkel and Rabbits, EMBO J., 1, 403-407, 1982.
24. Kramer et al. Nuc.Acids Res., 12, 9441-9446, 1984.
25. Hieter et al., Cell, 22, 197-207, 1980.
26. Lopata et al., Nuc.Acids Res., 12, 5707-5717, 1984.
27. Whittle et al., Prot. Eng., 1, 6, 499-505, 1987.

- 34 -

CLAIMS

1. A humanised antibody molecule (HAM) having specificity for the TAG-72 antigen and having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domains are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin.
2. The HAM of claim 1, wherein the entire variable domains are derived from the B72.3 MAb.
3. The HAM of claim 1 or claim 2, when produced by recombinant DNA technology.
4. The HAM of any one claims 1 to 3, which comprises a complete antibody molecule, an Fab fragment or an (Fab')₂ fragment.
5. The HAM of any one of claims 1 to 4, wherein an effector or reporter molecule is attached thereto.
6. The HAM of claim 5, wherein the effector or reporter molecule is a protein molecule which is coexpressed as a fusion protein with one of the chains of the HAM.
7. A process for producing the HAM of any one of claims 1 to 6, which process comprises
 - (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain wherein at least the

- 35 -

CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain wherein at least the CDRs of the variable domain are derived from the B72.3 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the HAM.

8. The process of claim 7, wherein the heavy and light chain encoding sequences are present on the same vector.

9. The process of claim 7, wherein the heavy and light chain encoding sequences are present on separate vectors.

10. The process of any one of claims 7 to 9, wherein the DNA coding sequences comprise fusions of cDNA and genomic DNA.

11. The process of claim 10, wherein the host cell is a non-myeloid mammalian cell.

1/13

FIG. 1

A

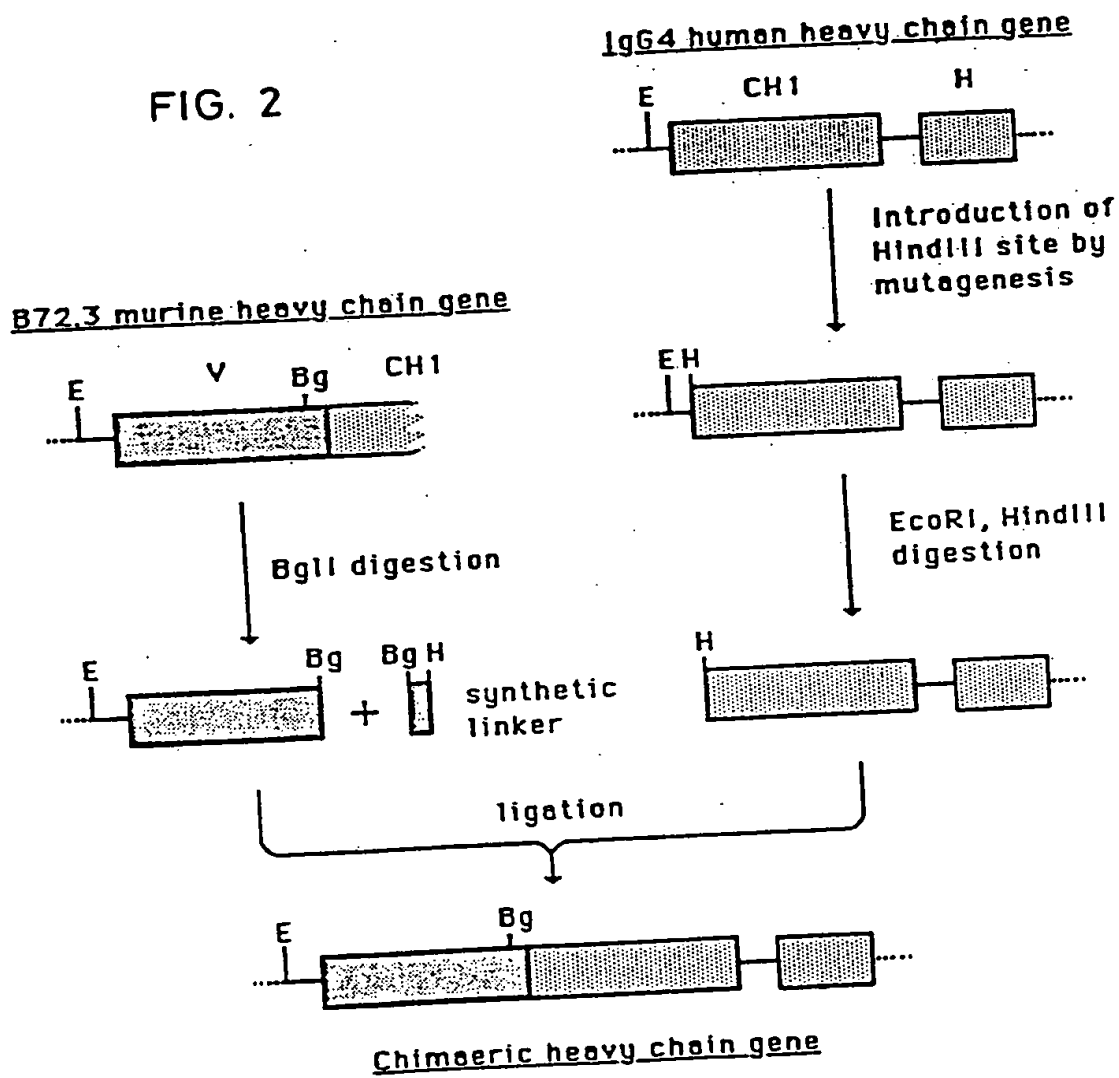
10 30 50
 GAATTCCTCACTGACTCTAACCATGGAATGGAGCTGGGTCTTTCTCTTCTCCTGTCAGTA
 MetGluTrpSerTrpValPheLeuPhePheLeuSerVal
 70 90 110
 ACTGACCTGCTCACTCCAGGTTTCAGCTGCAGCAGTCTGACGCTGAGTTGGTGAAACCT
 ThrGlyValHisSerGlnValGlnLeuGlnGlnSerAspAlaGluLeuValLysPro
 130 150 170
 GGGGCTTCCTGTAACATATCCTGCAAGGCTTCTGGCTACACCTTCACTGACCATGCTATT
 GlyAlaSerValLysIleSerCysLysAlaSerGlyTyrThrPheThrAspHisAlaIle
 190 210 230
 CACTGGGCGAAGCAGAAGCCTGAACAGGGCCTGGAATGGATTGGATATATTTCTCCCGGA
 HisTrpAlaLysGlnLysProGluGlnGlyLeuGluTrpIleGlyTyrIleSerProGly
 250 270 290
 AATGATGATATTAAGTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACTGCAGACAAA
 AsnAspAspIleLysTyrAsnGluLysPheLysGlyLysAlaThrLeuThrAlaAspLys
 310 330 350
 TCCTCCAGCACTGCCTACATGCAGCTCAACAGCCTGACATCTGAGGATTCTGCAGTGTAT
 SerSerSerThrAlaTyrMetGlnLeuAsnSerLeuThrSerGluAspSerAlaValTyr
 370 390 410
 TTCTGTAAAAGATCGTACTACGGCCACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
 PheCysLysArgSerTyrTyrGlyHisTrpGlyGlnGlyThrThrLeuThrValSerSer

B

10 30 50
 ATCACACACACACATGAGTGTGCCCCTCAGGTCCTGGGGTTGCTGCTGCTGTGGCTT
 MetSerValProThrGlnValLeuGlyLeuLeuLeuLeuTrpLeu
 70 90 110
 ACAGATGCCAGATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGTATCTGTG
 ThrAspAlaArgCysAspIleGlnMetThrGlnSerProAlaSerLeuSerValSerVal
 130 150 170
 GGAGAAACTGTCACCATCACATGTCGAGCAAGTGAGAATATTTACAGTAATTTAGCATGG
 GlyGluThrValThrIleThrCysArgAlaSerGluAsnIleTyrSerAsnLeuAlaTrp
 190 210 230
 TATCAACAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATGCTGCAACAACTTAGCA
 TyrGlnGlnLysGlnGlyLysSerProGlnLeuLeuValTyrAlaAlaThrAsnLeuAla
 250 270 290
 GATGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCGGGCACACAGTATTCCTCAAGATC
 AspGlyValProSerArgPheSerGlySerGlySerGlyThrGlnTyrSerLeuLysIle
 310 330 350
 AACAGCCTGCAGTCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTGGGGTACTCCG
 AsnSerLeuGlnSerGluAspPheGlySerTyrTyrCysGlnHisPheTrpGlyThrPro
 370 390 410
 TACACGTTCCGAGGGGGGACCAGGCTGGAAATAAAACGGGCTGATGCTGCACCAACTGTC
 TyrThrPheGlyGlyGlyThrArgLeuGluIleLysArgAlaAspAlaAlaProThrVal

2/13

FIG. 2



SUBSTITUTE SHEET

3/13

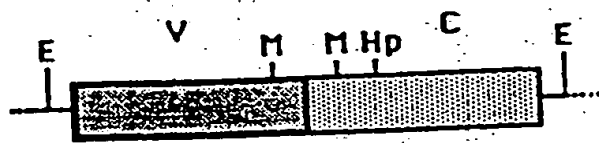
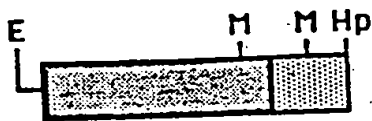
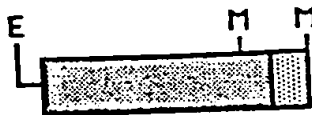
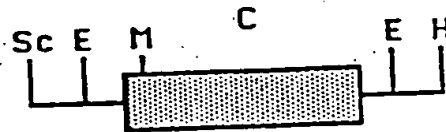
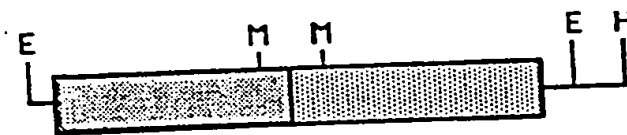
B72.3 murine light chain geneEcoRI, Hpa I
digestionMbolI partial
digestion

FIG. 3

Human K light chain geneMbolI
digestion

ligation

Chimeric light chain gene

SUBSTITUTE SHEET

4/13

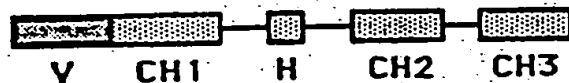
1 Murine heavy chain cDNA



2 Murine light chain cDNA



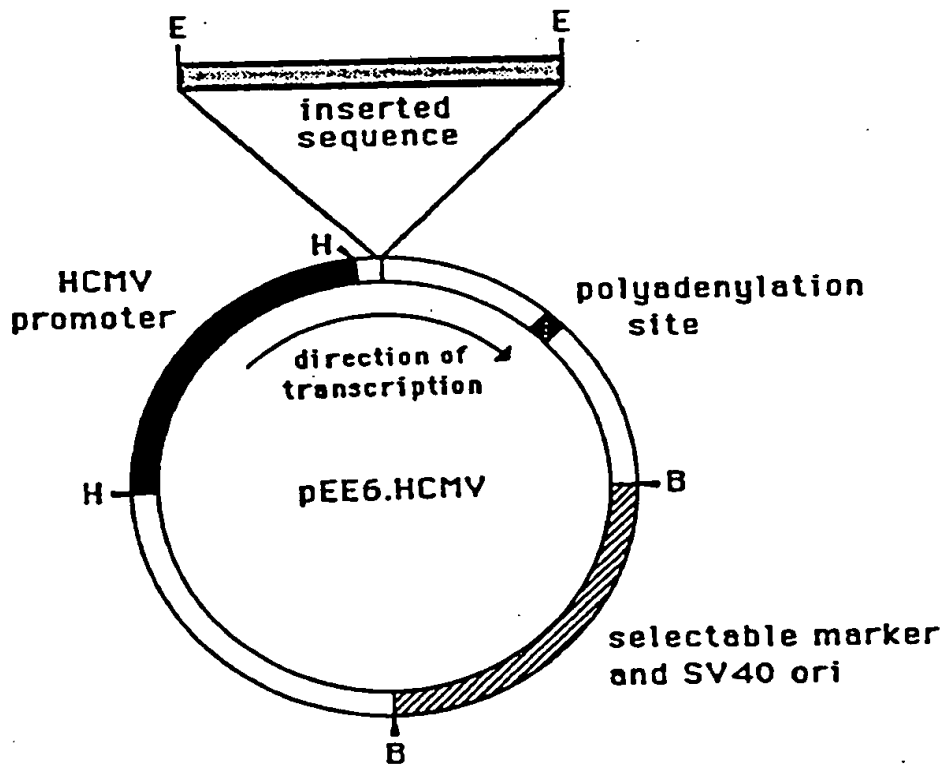
3 Chimaeric heavy chain gene



4 Chimaeric light chain gene



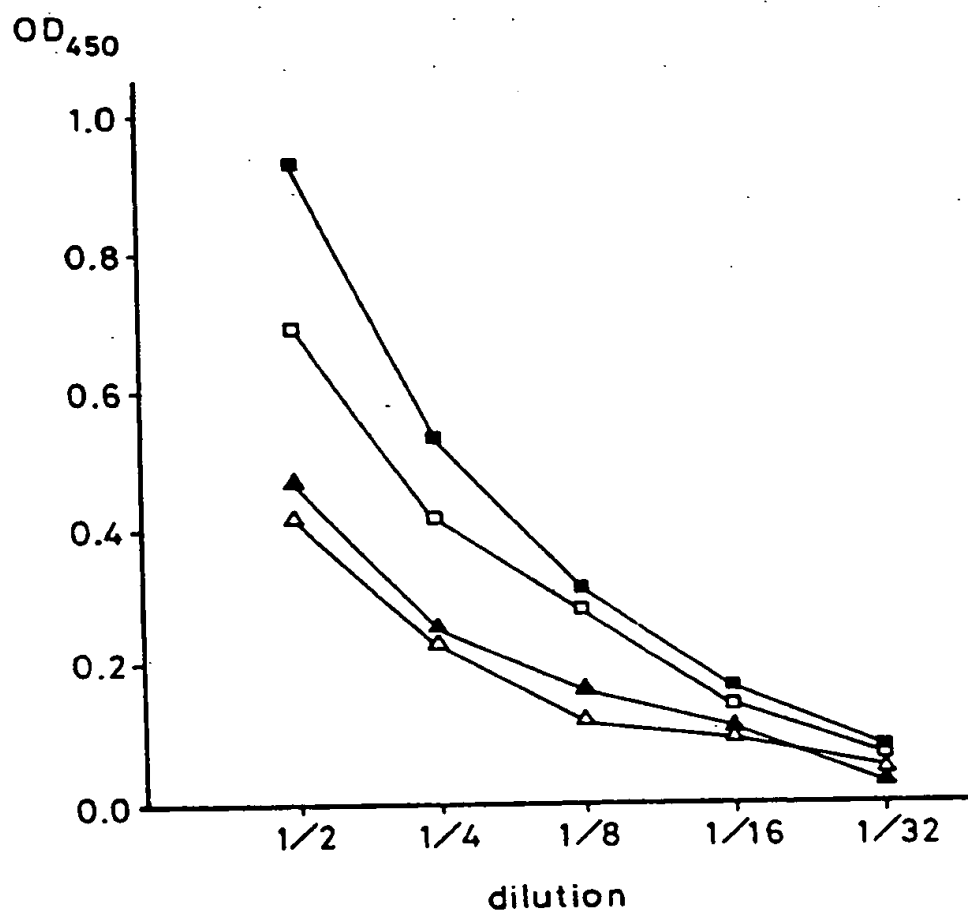
FIG. 4



SUBSTITUTE SHEET

5/13

FIG. 5



SUBSTITUTE SHEET

6/13

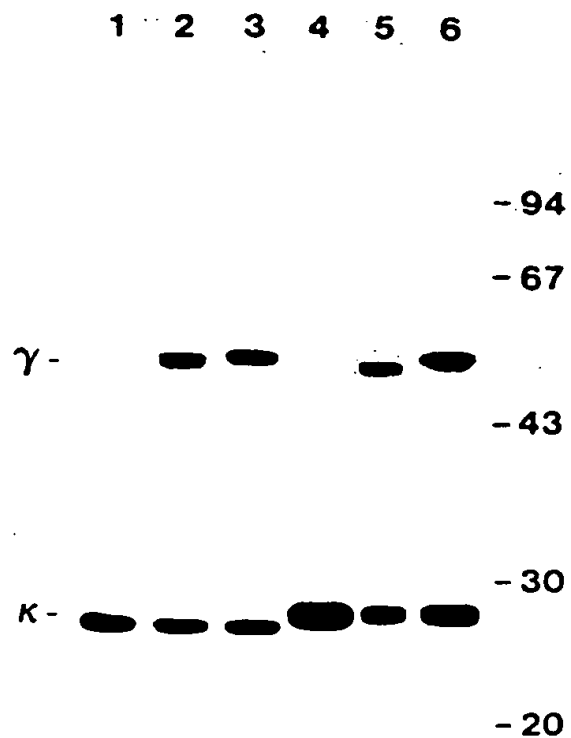


FIG. 6

7/13

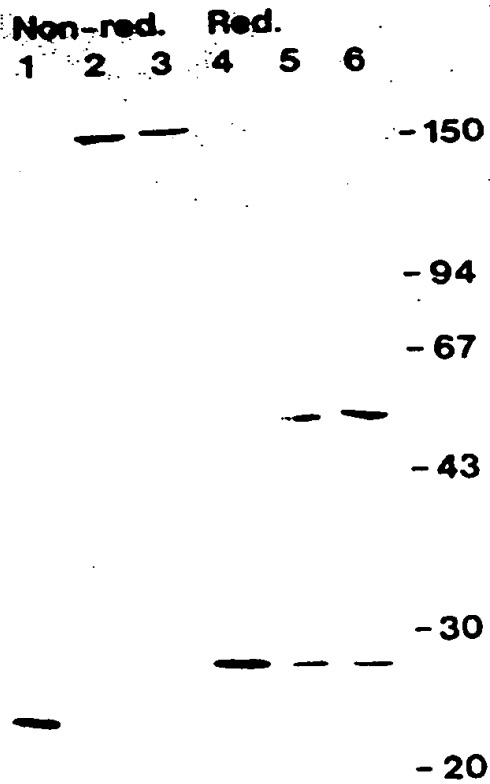


FIG. 7

8/13

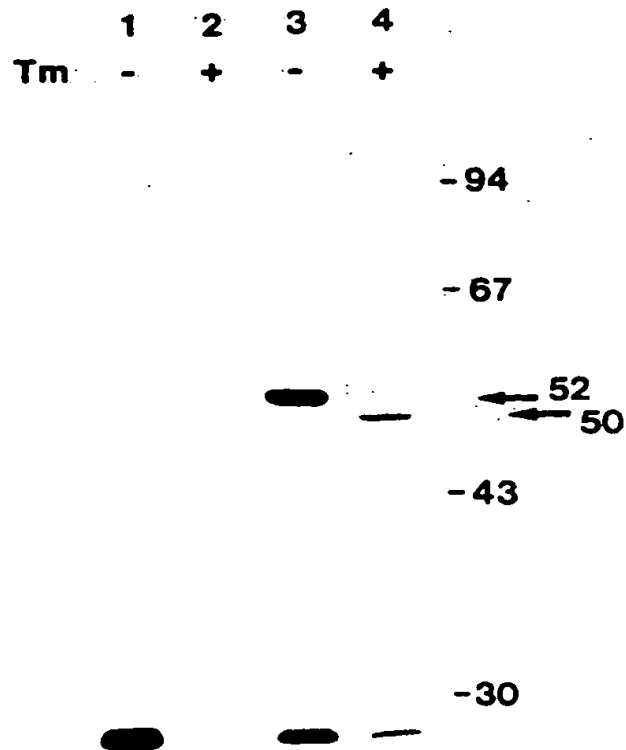


FIG. 8

SUBSTITUTE SHEET

9/13

SDS-PAGE OF CHIMAERIC B72.3

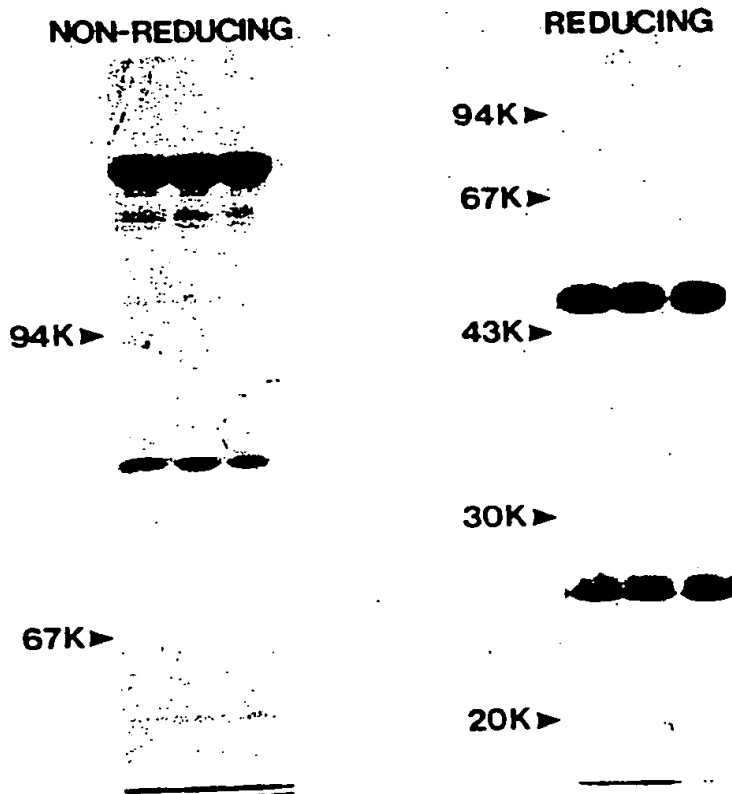
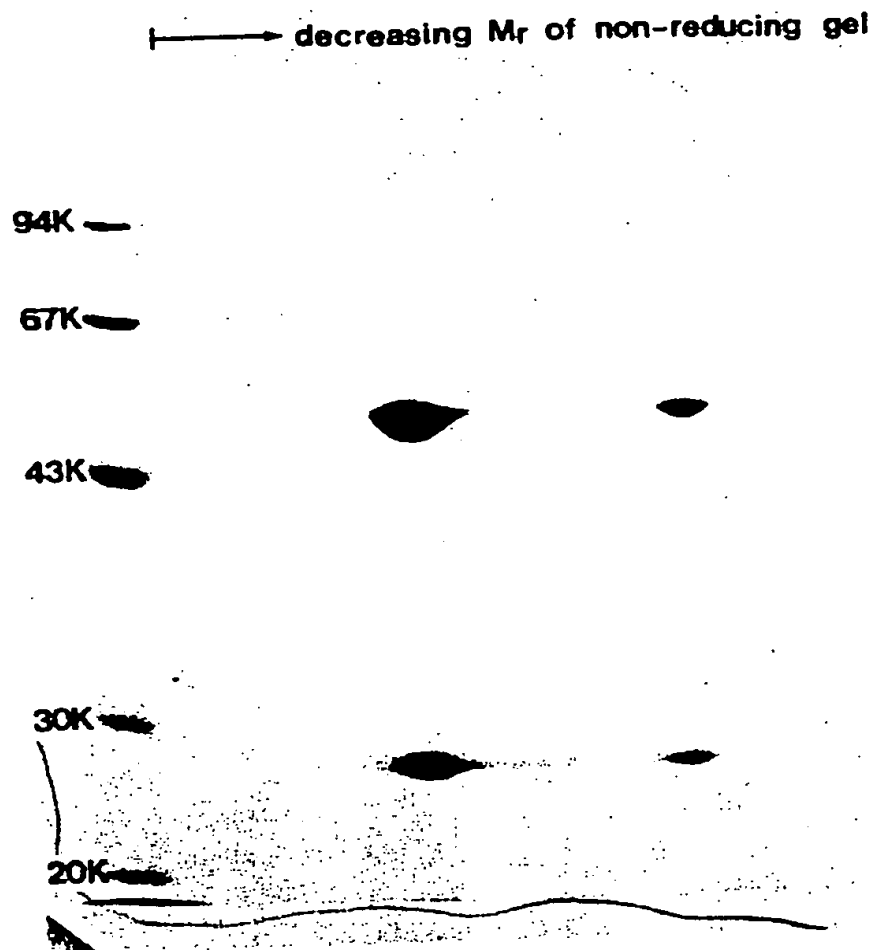


FIG. 9

10/13

2-DIMENSIONAL SDS-PAGE OF CHIMAERIC B72.3

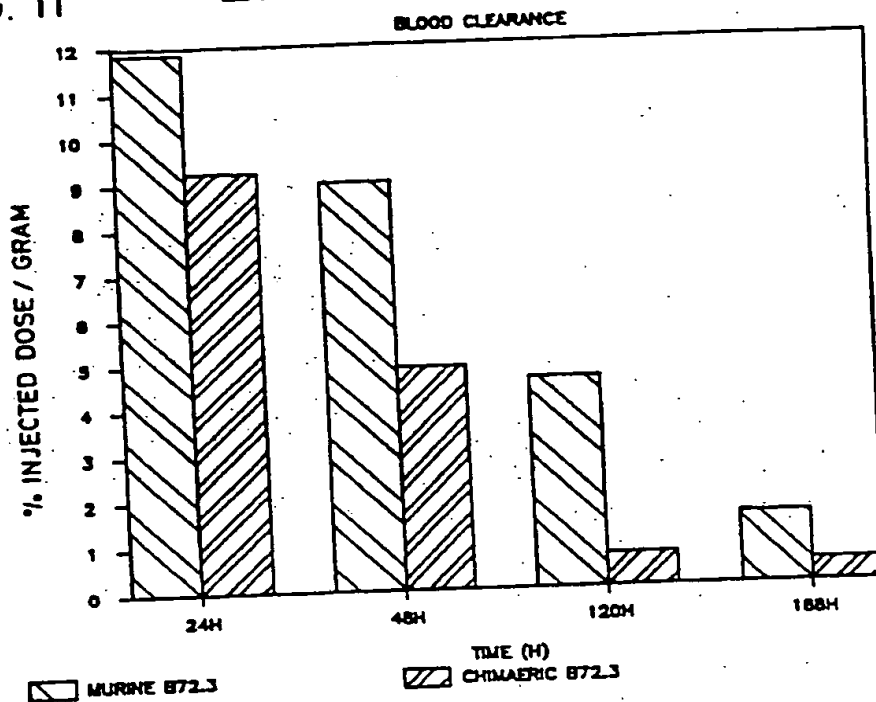
1st dimension: non-reducing SDS-PAGE
2nd dimension: reducing SDS-PAGE

FIG. 10

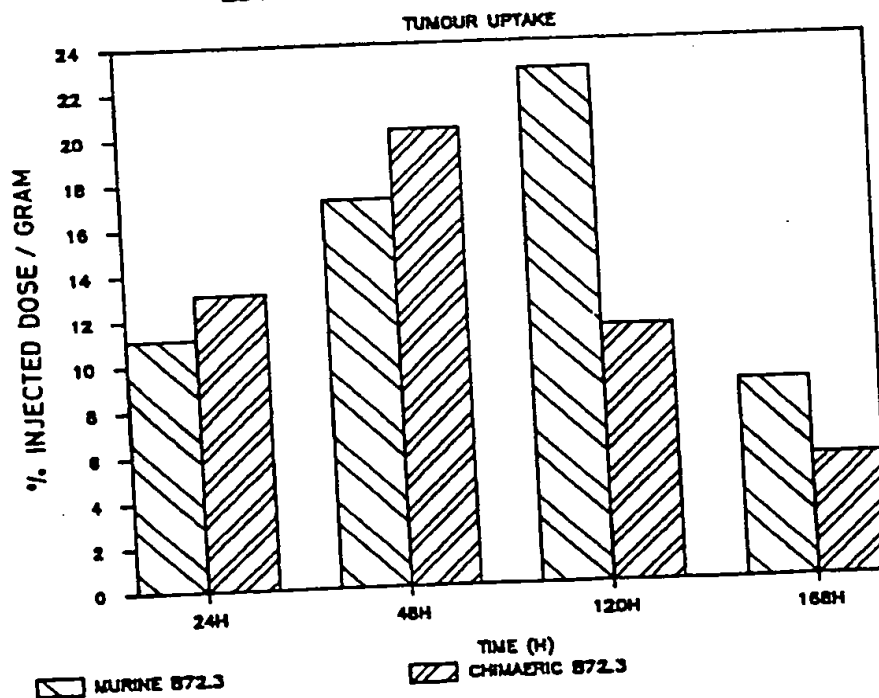
SUBSTITUTE SHEET

FIG. 11

LS174T XENOGRAFT: CHIMAERIC B72.3



LS174T XENOGRAFT: CHIMAERIC B72.3

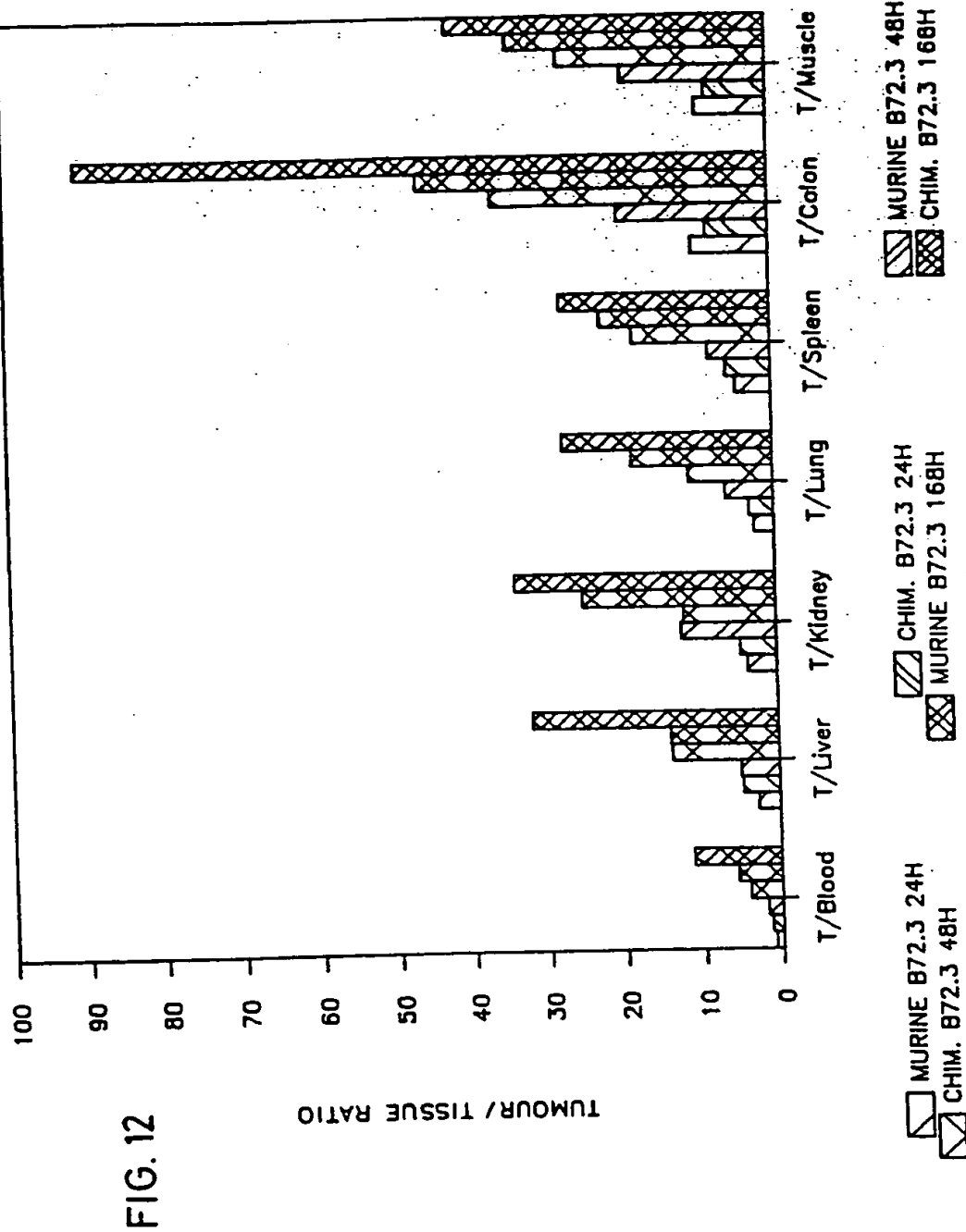


SUBSTITUTE SHEET

12/13

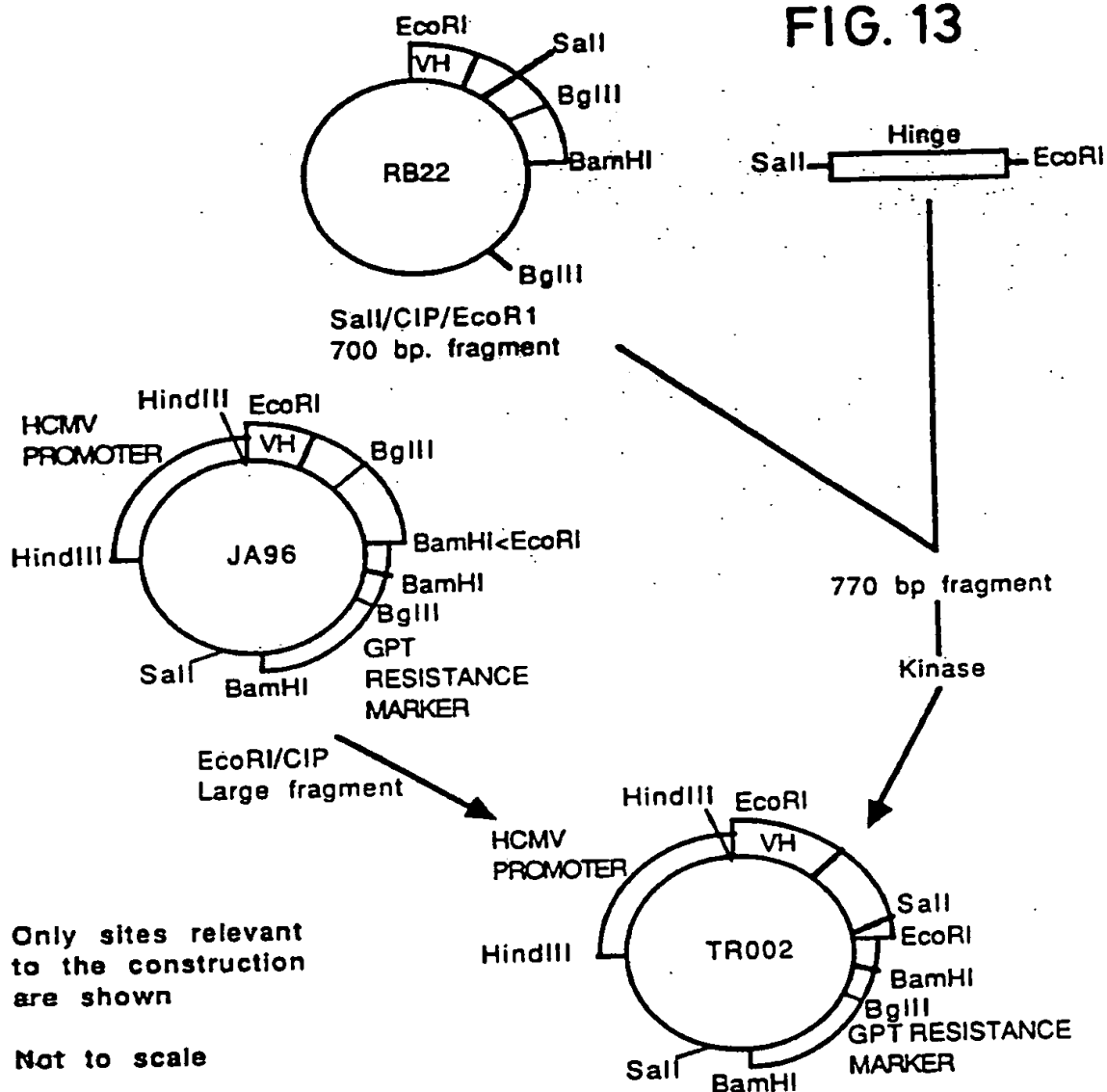
CHIMAERIC B72.3 v MURINE B72.3

TUMOUR/TISSUE RATIOS: 24-168H



13/13

FIG. 13



CONSTRUCTION OF TR002
B72.3VH/HUMAN IgG1 F(ab') CONSTANT
cDNA LIKE CHIMAERIC HEAVY CHAIN (γ1 HINGE)
IN EE6 BASED EXPRESSION VECTOR

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.